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Direct action of endocrine disrupting chemicals on human sperm

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Abstract

Synthetic endocrine disrupting chemicals (EDCs), omnipresent in food, household, and personal care products, have been implicated in adverse trends in human reproduction, including infertility and increasing demand for assisted reproduction. Here, we study the action of 96 ubiquitous EDCs on human sperm. We show that structurally diverse EDCs activate the sperm-specific CatSper channel and, thereby, evoke an intracellular Ca^{2+} increase, a motility response, and acrosomal exocytosis. Moreover, EDCs desensitize sperm for physiological CatSper ligands and cooperate in low-dose mixtures to elevate Ca^{2+} levels in sperm. We conclude that EDCs interfere with various sperm functions and, thereby, might impair human fertilization.

Keywords Ca^{2+} signalling; CatSper; endocrine disrupting chemical; human sperm

Subject Categories Chemical Biology; Membrane & Intracellular Transport; Physiology

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Introduction

In mammalian sperm, CatSper represents the principal Ca^{2+} channel, controlling intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) and motility [1–4]. Male mice lacking CatSper are infertile, because CatSper$^{-/-}$ sperm fail to undergo rheotaxis and hyperactivation [1,5]. Mutations in CatSper genes have been correlated with male infertility [6,7]. In human sperm, progesterone and prostaglandins, two hormones released into the oviduct [8], directly activate CatSper [2,9]. Progesterone- and prostaglandin-induced Ca^{2+} influx has been suggested to control sperm capacitation, chemotaxis, hyperactivation, and acrosomal exocytosis [10–12].

In fact, human CatSper serves as a polymodal chemosensor that harbors promiscuous binding sites for structurally diverse ligands: *In vitro*, CatSper is directly activated by hydrophobic agents like synthetic odorants that can mimic the action of female ligands [13]. Moreover, CatSper is also activated by p,p′-DDE, a metabolite of dichlorodiphenyltrichloroethane (DDT) [14]. These observations indicate that EDCs in reproductive fluids might commonly interfere with human sperm function.

EDCs mimic the action of hormones and affect their production or metabolism. EDCs have been linked to decreasing fertility rates in the Western world [15,16], testis cancer, and widespread infertility [16–19] (see also Sharpe RM, 2012; DOI 10.1038/embor.2012.50). However, due to the lack of appropriate human models, the actions of EDCs are debated.

Here, we systematically study the action on human sperm of ubiquitous EDCs, including biocides, plasticizers, components of personal care products, surfactants, pharmaceuticals, phytoestrogens, and polychlorinated biphenyls (Fig 1A, Supplementary Table S1). We show by Ca^{2+} fluorimetry, patch-clamp recordings, and motility analysis that structurally diverse EDCs, at concentrations present in human body fluids, directly activate CatSper and, thereby, interfere with various sperm functions. Our findings substantiate common concerns regarding the negative impact of EDCs on male reproductive health and should be considered for future regulations toward a more restrictive use of EDCs.

Results

Structurally diverse EDCs evoke Ca^{2+} responses in human sperm

Using 384-microtiter plates, we monitored $[Ca^{2+}]_i$ in human sperm. Injection of progesterone into the wells evoked a rapid, transient increase in $[Ca^{2+}]_i$ followed by a slow, sustained elevation [2,20]; buffer injection produced only a small mixing artifact (Fig 1B and C,

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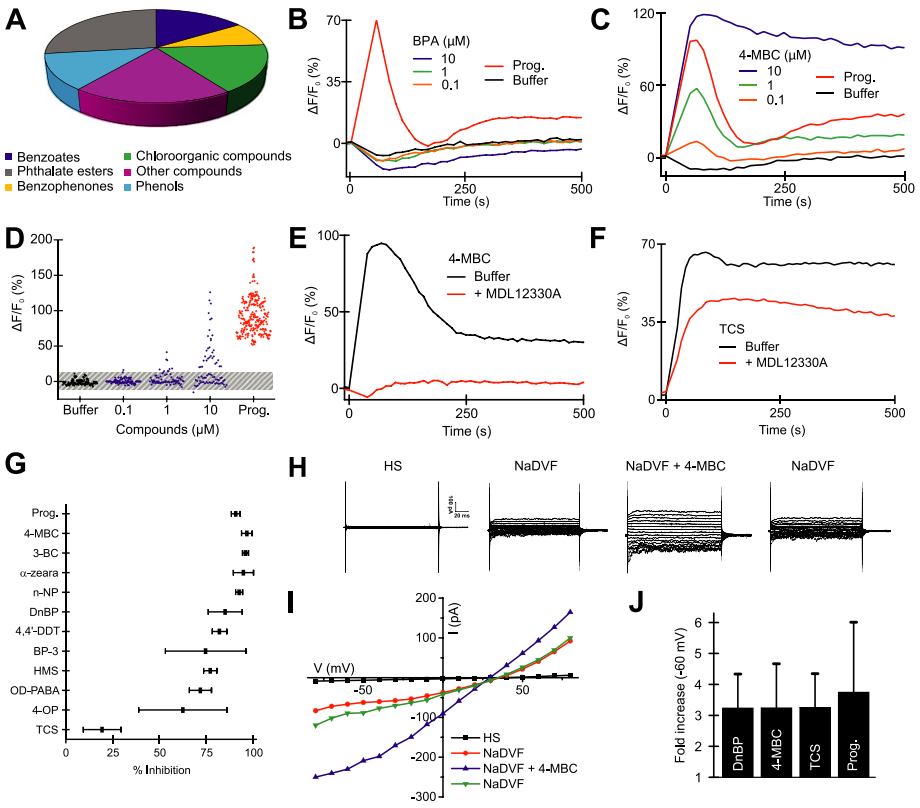

Figure 1. Structurally diverse EDCs directly activate CatSper in human sperm.

A Composition of the compound library comprising 96 EDCs.

B Bisphenol A (BPA) does not evoke Ca^{2+} signals in human sperm. $\Delta F/F_0$ (%) indicates the percentage change in fluorescence (ΔF) with respect to the mean basal fluorescence (F_0) before the application of buffer, progesterone (2 µM) or chemicals.

C 4-Methylbenzylidene camphor (4-MBC)-evoked Ca^{2+} signals in human sperm; progesterone = 2 µM.

D Mean (n = 4–6) Ca^{2+} signal amplitudes evoked by EDCs at 0.1, 1, and 10 µM (blue), compared to signal amplitudes evoked by progesterone (2 µM, red) and buffer (black). Shaded area: buffer ± 3 SD; EDCs that evoked amplitudes > mean buffer + 3 SD were defined as 'active'.

E 4-MBC-induced Ca^{2+} signals (10 µM) in the absence (buffer) and presence of the CatSper inhibitor MDL12330A (100 µM).

F Triclosan (TCS)-induced Ca^{2+} signals (10 µM) in the absence (buffer) and presence of the CatSper inhibitor MDL12330A (100 µM).

G Relative inhibition of EDC (3-30 µM)- and progesterone (2 µM)-induced Ca^{2+} signals by MDL12330A (100 µM) [TCS, 4-octylphenol (4-OP), benzophenone-3 (BP-3), 4,4′-DDT, n-nonylparaben (n-NP), 4-MBC: n = 3; progesterone, 3-benzylidene camphor (3-BC), α-zearalenol, di-n-butyl phthalate (DnBP), homosalate (HMS), padimate O (OD-PABA): n = 4].

H 4-MBC (10 µM) reversibly enhanced monovalent whole-cell CatSper currents (NaDVF + 4-MBC) in human sperm, recorded in Na^+-based divalent-free solution (NaDVF), in the absence of intracellular divalent ions. Voltage was stepped from 0 ± 80 mV in increments of 10 mV. HS: currents recorded in the presence of extracellular Ca^{2+} and Mg^{2+} .

I Current-voltage relation of currents shown in (H).

J Increase in monovalent CatSper currents at −60 mV evoked by DnBP (100 µM; n = 4), 4-MBC (10 µM; n = 9), TCS (10 µM; n = 4), and progesterone (2 µM; n = 6).

Data information: All values are given as mean ± SD.

Supplementary Fig S1A). We analyzed the progesterone and buffer responses to determine the assay's Z′-factor, a statistical parameter for the reliability of screening assays [21]. We obtained a Z′-factor of 0.79 ± 0.13 (n = 33) (mean ± SD, n = number of experiments) (Supplementary Fig S1B), demonstrating that the assay reliably differentiates between 'active' and 'inactive' chemicals.

Along with buffer and progesterone controls, EDCs (Fig 1A, Supplementary Table S1) were tested at concentrations of 0.1, 1, and 10 µM (n = 4–6); a few chemicals were tested only at 0.1 µM. The efficacy of EDCs to evoke a Ca^{2+} signal in sperm was wide ranging. For example, the plasticizer bisphenol A (BPA) did not affect $[Ca^{2+}]_i$ (Fig 1B). In contrast, 4-methylbenzylidene camphor (4-MBC), a UV-filter, evoked a rapid biphasic Ca^{2+} increase at 0.1 and 1 µM, whereas at 10 µM, the Ca^{2+} signal was more sustained (Fig 1C). The signal amplitude increased in a dose-dependent fashion. Figure 1D shows the mean amplitude of Ca^{2+} signals evoked by 96 EDCs (see Supplementary Table S1). Sixty-three EDCs did not affect $[Ca^{2+}]_i$, that is, signal amplitudes were similar to that of buffer controls (Fig 1D, shaded area). However, 33 EDCs evoked a sizeable Ca^{2+} response at 10 µM; for several EDCs, Ca^{2+} amplitudes were similar to those evoked by progesterone (Fig 1D). Moreover, 12 of the 33 EDCs evoked Ca^{2+} signals at 1 µM; 4-MBC and the insecticide 4,4′-DDT evoked Ca^{2+} signals even at 0.1 µM (Fig 1D, Supplementary Table S1). In conclusion, about 30% of ubiquitous EDCs increase Ca^{2+} levels in human sperm.

Table 1. Summary of data obtained for the 11 EDCs selected for in-depth analysis

Trivial name	IUPAC name (PubChem)	Common abbreviation	Common use	EC_{50} ± SD (μM)	EC_{02} ± SD (nM)	Inhibition by MDL12330A (%)	Max. observed concentration in human blood, serum, or plasma
3-(4-Methyl benzylidene) camphor	4,7,7-Trimethyl-2-[(4-methyl phenyl) methylidene] bicyclo[2.2.1]heptan-3-one	4-MBC	UV-filter used in sunscreens and personal care products	6.83 ± 2.26 (n = 4)	107.3 ± 115.5 (n = 4)	96.64 ± 2.83 (n = 3)	*318 nM (plasma) [28]
3-Benzylidene camphor/benzal camphor	2-Benzylidene-4,7,7-trimethyl bicyclo[2.2.1]heptan-3-one	3-BC	UV-filter used in sunscreens and personal care products	1.73 ± 1.36 (n = 3)	21.6 ± 28.3 (n = 3)	95.88 ± 1.63 (n = 4)	No human blood concentration published
4-Octylphenol	4-Octylphenol	4-OP	Industrial chemical, e.g. used as a precursor of surfactants, or to produce phenolic resins for tire manufacturing	5.93 ± 0.40 (n = 3)	744.7 ± 420.3 (n = 4)	62.49 ± 23.49 (n = 3)	No human blood concentration published
α-Zearalenol	(2E,7R,11S)-7,15,17-Trihydroxy-11-methyl-12-oxabicyclo[12.4.0] octadeca-1(14),2,15, 17-tetraen-13-one	α-zeara	Estrogenic derivative of the mycotoxin zeranol used as hormonal growth promoter in livestock industry	1.72 ± 1.12 (n = 5)	30.9 ± 25.8 (n = 5)	94.77 ± 5.46 (n = 4)	1.19 ± 2.80 μM (serum) [27]
Benzophenone-3/ oxybenzone	(2-Hydroxy-4-methoxyphenyl)-phenylmethanone	BP-3	UV-filter used in sunscreens and personal care products	23.69 ± 15.56 (n = 6)	537.4 ± 453.6 (n = 4)	74.60 ± 21.45 (n = 3)	*3.49 μM (plasma) [28]
Chlorophenothane	1-Chloro-4-[2,2,2-trichloro-1-(4-chlorophenyl)ethyl]benzene	4,4′-DDT/ p,p′-DDT	Insecticide used for malaria control	< 1	~ 0.3	82.00 ± 3.96 (n = 3)	611 nM whole blood [26]; 9.59 and 8.38 nM (maternal and cord serum, respectively) [39]
Dibutyl phthalate/ di-n-butyl phthalate	Dibutyl benzene-1,2-dicarboxylate	DnBP	Common plasticizer in food containers and beverage bottles	19.91 ± 6.45 (n = 5)	1,000 ± 800 (n = 4)	85.05 ± 9.15 (n = 4)	32.69 nM (blood) [30]
Homosalate/ eusolex	(3,3,5-Trimethylcyclohexyl) 2-hydroxybenzoate	HMS	UV-filter used in sunscreens and personal care products	5.70 ± 1.54 (n = 3)	232.1 ± 133.1 (n = 3)	77.11 ± 3.50 (n = 4)	No human blood concentration published
Nonylparaben/ 4-hydroxybenzoic acid n-nonyl ester	Nonyl 4-hydroxybenzoate	n-NP	Antibacterial and antifungal biocide used as preservative for food and cosmetics	3.13 ± 1.27 (n = 3)	189.7 ± 122.5 (n = 3)	92.51 ± 1.84 (n = 3)	No human blood concentration published
Padimate O	2-Ethylhexyl 4-(dimethylamino) benzoate	OD-PABA	UV-filter used in sunscreens and personal care products	2.53 ± 1.72 (n = 3)	67.0 ± 58.5 (n = 3)	71.81 ± 5.96 (n = 4)	No human blood concentration published
Triclosan/irgasan	5-Chloro-2-(2,4-dichlorophenoxy) phenol	TCS	Antibacterial and antifungal biocide present in many consumer and personal care products, e.g. toys and toothpastes	8.44 ± 4.80 (n = 5)	283.4 ± 267.5 (n = 5)	19.36 ± 10.14 (n = 3)	31.7 nM (serum) [40]; 55 nM (plasma) [31]

For each compound, trivial name, IUPAC name, the common abbreviation, common use, EC_{50} value, EC_{02} value, and the degree of inhibition by MDL12330A are indicated. If available, published concentrations in human blood are provided as maximal (max) and mean values.

EDCs directly activate CatSper

We unraveled the underlying mechanism for 11 selected EDCs with diverse chemical structures (Table 1, Supplementary Fig S1C). We used the CatSper inhibitor MDL12330A (MDL) [13] to examine whether EDC-induced Ca^{2+} signals involve CatSper. MDL abolished Ca^{2+} signals evoked by 4-MBC, 3-benzylidene camphor (3-BC), α-zearalenol, and nonylparaben (n-NP) (Fig 1E and G); Ca^{2+} signals evoked by padimate O (OD-PABA), di-*n*-butyl phthalate (DnBP), benzophenone-3 (BP-3), homosalate (HMS), and 4,4′-DDT were suppressed by 70–80% (Fig 1G). We conclude that these chemicals primarily act via activation of CatSper. Of note, triclosan (TCS)-evoked Ca^{2+} signals were suppressed only by 20–25% (Fig 1F and G), indicating that TCS activates CatSper and, in addition, releases Ca^{2+} from intracellular stores [22,23] or inhibits Ca^{2+} export by Ca^{2+}-ATPase or Na^+/Ca^{2+} exchange.

CatSper is sensitive to the intracellular pH (pH_i) [2,3,9]. Except for n-NP, none of the EDCs evoked sizeable changes in pH_i of human sperm (Supplementary Fig S2A), excluding the possibility that chemicals activate CatSper via cellular alkalization. n-NP evoked a slow, sustained alkalization (Supplementary Fig S2A and B), suggesting that the sustained component of the n-NP-evoked Ca^{2+} signal is due to CatSper activation at alkaline pH_i (Supplementary Fig S2B).

We scrutinized by an independent technique that EDCs directly activate CatSper: Using whole-cell patch-clamp recordings from human sperm, we studied the action of 4-MBC, DnBP, and TCS on CatSper currents. In standard extracellular solution containing Ca^{2+} and Mg^{2+}, only small or no currents were evoked by stepping the membrane voltage (V_m) from 0 ± 80 mV (Fig 1H and I; HS). Characteristic monovalent CatSper currents were recorded in Na^+-based divalent-free extracellular solution (Fig 1H and I; NaDVF) [2,3,9,13]. These monovalent currents are abolished in sperm that lack CatSper [24]. Similar to progesterone, 4-MBC, DnBP, and TCS reversibly enhanced monovalent currents by about threefold (−60 mV) (Fig 1H–J, Supplementary Fig S2C and D). The reversal potential of basal CatSper currents, progesterone-evoked CatSper currents, and EDC-evoked currents was indistinguishable (Fig 1I, Supplementary Fig S2C and D). Moreover, currents evoked by simultaneous stimulation of sperm with progesterone and 4-MBC were abolished by the CatSper inhibitor MDL (Supplementary Fig S2E). We conclude that structurally diverse EDCs directly activate CatSper.

EDCs compete with physiological ligands for CatSper activation

To examine whether EDCs compete with progesterone or prostaglandins for CatSper activation, we studied cross-desensitization between EDCs (4-MBC, α-zearalenol, and TCS) and progesterone/prostaglandin E1 (PGE1). Progesterone and prostaglandins employ distinct binding sites to activate CatSper [2,9,12]. Accordingly, 3-CMO-progesterone, but not PGE1, cross-desensitized sperm for progesterone: 3-CMO-progesterone increased the constant of half-maximal activation (EC_{50}) for progesterone from 20 nM to about 150 nM (Fig 2B, upper panel). In contrast, PGF1α, but not progesterone, increased the EC_{50} for PGE1 from 6 nM to about 15 nM (Fig 2D, upper panel). TCS did not affect the EC_{50} for progesterone or PGE1 (Fig 2B and D, lower panels), indicating that TCS activates CatSper via a distinct mechanism. However, 4-MBC increased the EC_{50} for progesterone, but not for PGE1, whereas α-zearalenol increased the EC_{50} for PGE1, but not for progesterone (Fig 2A–D). Monovalent CatSper currents evoked by saturating progesterone concentrations (1 μM) were only slightly enhanced by 4-MBC (10 μM) (Supplementary Fig S2E). Altogether, we conclude that EDCs compete with progesterone and prostaglandins for CatSper activation and, thereby, desensitize sperm for these physiological ligands. Of note, we cannot exclude the possibility that EDCs also employ hitherto unknown sites or mechanisms to activate CatSper.

EDCs evoke motility responses and acrosomal exocytosis

We further tested whether progesterone and EDCs evoke similar behavioral responses in sperm by analyzing the frequency and asymmetry of the flagellar beat in head-tethered sperm. At rest, the beat frequency was 28.5 ± 9.9 Hz and the beating pattern was symmetrical (asymmetry: 0.09 ± 0.06 rad; $n = 15$) (Fig 3A); perfusion with buffer did not affect the beating pattern (Fig 3A and B). However, perfusion with 4-MBC or progesterone (Supplementary Movies S1 and S2) rapidly lowered the frequency and enhanced the asymmetry of the beat in a dose-dependent fashion (Fig 3A and B, Supplementary Fig S3). Asymmetric beating is a hallmark of sperm hyperactivation [25]. Furthermore, we examined whether 4-MBC, 3-BC, and TCS stimulate acrosomal exocytosis. Both progesterone and these chemicals evoked acrosomal exocytosis in 25–40% of sperm (Fig 3C). We conclude that CatSper activation by EDCs rapidly changes sperm motility and stimulates acrosomal exocytosis.

EDCs act at physiologically relevant concentrations

Finally, we examined whether EDCs activate CatSper at concentrations reached in body fluids. To this end, the potency and lowest-effective dose for the 11 selected EDCs was quantified. For example, analysis of Ca^{2+} signals evoked by α-zearalenol (Fig 4A) yielded an EC_{50} of 1.7 μM (Fig 4B and C, Table 1). The EC_{50} values for the EDCs ranged between 1.7 and 23.7 μM (Fig 4B and C, Table 1). Thus, EDCs are at least 100-fold less potent than progesterone and PGE1 to activate CatSper (Fig 4B). The EC_{50} for 4,4′-DDT was only estimated, because Ca^{2+} signals did not saturate at concentrations for which this chemical was soluble (Supplementary Fig S4). From the dose-response relationships, we yielded the lowest-effective concentrations (EC_{02}) (Fig 4B), which ranged between 30 and 1,000 nM (Fig 4C, gray triangles, Table 1). For 4,4′-DDT, the estimated EC_{02} was about 300 pM (Fig 4D), similar to the previously reported potency of p,p′-DDE [14].

Only few data on EDC concentrations in body fluids exist (Table 1). The maximal concentrations of 4,4′-DDT, α-zearalenol, BP-3, and 4-MBC in blood are similar or higher than the respective EC_{02} values (red versus gray triangles, Fig 4C) [26–28]. Up to 4 μM of n-NP was detected in urine, thus exceeding its EC_{02} [29]. The blood concentrations of TCS and DnBP are 1–2 orders of magnitude lower than their EC_{02} [30,31]; in seminal fluid, a DnBP concentration of ≈ 50 μM has been reported [32]. Together, these data indicate that EDCs affect $[Ca^{2+}]_i$ in human sperm at 'physiological' concentrations [14].

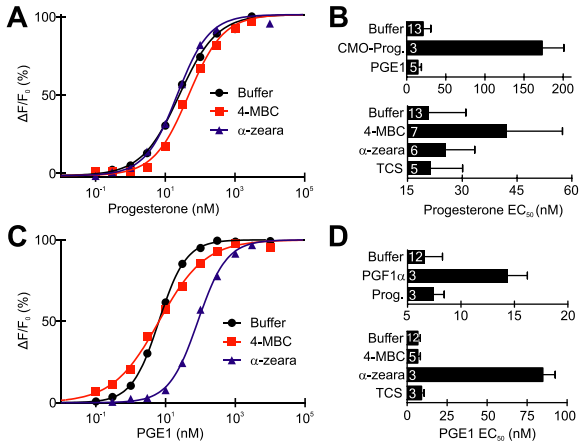

Figure 2. EDCs compete with physiological ligands for CatSper activation.

A Representative dose-response relationship of progesterone in the absence (buffer) and presence of 4-MBC (10 μM) or α-zearalenol (10 μM).

B Mean EC_{50} values of progesterone in the absence (buffer) and presence (upper panel) of 3-CMO-progesterone (100 nM) or prostaglandin E1 (PGE1; 100 nM), and in the presence (lower panel) of 4-MBC (10 μM), α-zearalenol (10 μM), or TCS (10 μM). The number of experiments is indicated in the bars.

C Representative dose-response relationship of PGE1 in the absence (buffer) and presence of 4-MBC (10 μM) or α-zearalenol (10 μM).

D Mean EC_{50} values of PGE1 in the absence (buffer) and presence (upper panel) of PGF1α (300 nM) or progesterone (100 nM) and in the presence (lower panel) of 4-MBC (10 μM), α-zearalenol (10 μM), or TCS (10 μM). The number of experiments is indicated in the bars.

Data information: All values are given as mean ± SD.

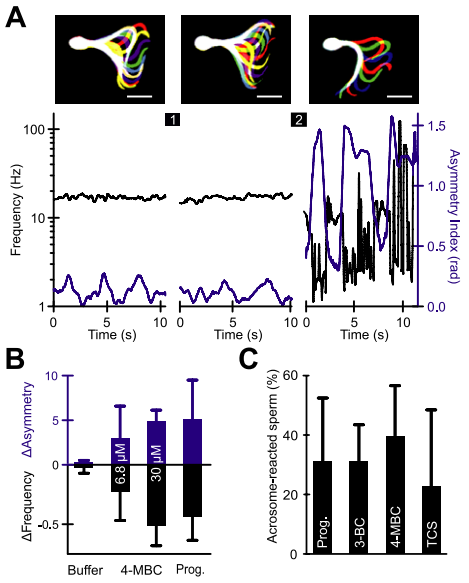

Figure 3. EDCs evoke motility responses and acrosomal exocytosis in human sperm.

A Images: Flagella waveform of head-tethered human sperm at rest (left, buffer) and following perfusion with buffer (middle) or 4-MBC (6.8 μM, right). Successive, aligned, and superimposed images yielding a quasi stop-motion image, illustrating one flagellar beating cycle (Scale bar: 15 μm). Graph: Time course of beat frequency (black) and flagellar asymmetry (blue) before and after perfusion with buffer and 4-MBC. An increase in the asymmetry index indicates a more asymmetric flagella waveform. (1) and (2) indicate perfusion of sperm with buffer and 4-MBC, respectively.

B Mean fold change in asymmetry index and beat frequency after perfusion with buffer (n = 15), 4-MBC [6.8 μM (n = 8) and 30 μM (frequency: n = 4, asymmetry: n = 3)], and progesterone (1 μM; n = 4).

C Percentage of acrosome-reacted sperm bathed in progesterone, 3-benzylidene camphor (3-BC), 4-MBC, and TCS (all 10 μM) relative to the vehicle (DMSO) control (all conditions: n = 5).

Data information: All values are given as mean ± SD.

EDCs cooperate to elevate Ca^{2+} levels

In vivo, sperm are presumably exposed to complex EDC mixtures [15] that might vary across the male and female genital tract. Therefore, we studied Ca^{2+} signals evoked by mixtures containing EDCs at their respective EC_{02} values: Ca^{2+} responses evoked by each EDC alone were miniscule, whereas the EDC mixture evoked a pronounced Ca^{2+} response (Fig 5A and B) that was similar to a Ca^{2+} response evoked by 10–30 nM progesterone (Fig 5C). Thus, EDCs cooperate to elevate sperm Ca^{2+} levels, suggesting that even low-dose EDC mixtures in body fluids affect human sperm *in vivo*.

Discussion

The EDC action on human CatSper could affect fertilization in several ways: Changes in $[Ca^{2+}]_i$ control sperm navigation across the female genital tract, hyperactivation, and acrosomal exocytosis [5,33–36]. Various physical and chemical cues provided across the oviduct assist sperm to coordinate these functions. EDCs in reproductive fluids might disturb the precisely coordinated sequence of events underlying fertilization: EDCs could evoke motility responses and acrosome reaction at the wrong time and wrong place; moreover, desensitization of sperm for female factors might hamper navigation toward the egg and penetration of its vestments. More data concerning EDC concentrations in seminal and oviductal fluids are required to strengthen and extend these conclusions. Of note, EDC action on sperm might be even more complex: Besides those EDCs that activate CatSper, other EDCs might inhibit rather than activate the channel.

Like the action of progesterone and prostaglandins [9], the EDC action on CatSper also seems to be specific for humans: In mouse sperm, CatSper-mediated Ca^{2+} signals were evoked by an alkaline/depolarizing medium (K8.6) [37] or the cGMP derivative 8-Br-cGMP [1,13] (Supplementary Fig S5). In contrast, α-zearalenol, 4-MBC, n-NP, DnBP, and 4,4′-DDT did not evoke Ca^{2+} responses (Supplementary Fig S5), demonstrating that mice are not a suitable model to study the EDC action on sperm and fertility.

The no-observed-adverse-effect-level (NOAEL) standard declares safety thresholds for individual EDCs. Our finding that EDCs cooperatively elevate Ca^{2+} levels challenges the validity of this standard procedure. To understand the action of EDC mixtures in mechanistic terms, it needs to be studied whether EDCs act additively or even synergistically.

Here, we provide a direct link between exposure to EDCs and potential adverse effects on fertilization in humans. About 800 omnipresent man-made chemicals are suspected to interfere with the endocrine system. To this day, the majority of these potential

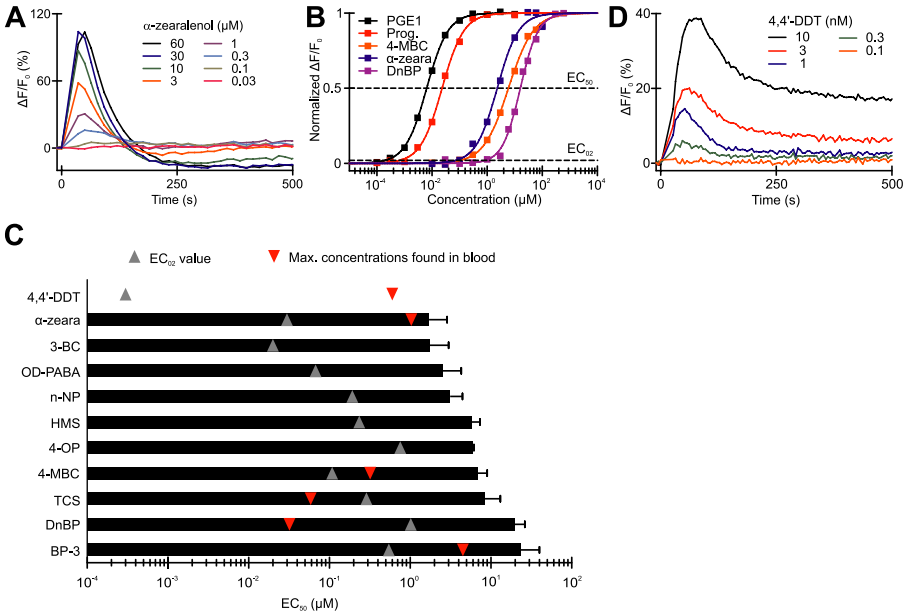

Figure 4. EDCs evoke Ca^{2+} responses at physiologically relevant concentrations.

A α-Zearalenol-evoked Ca^{2+} signals.

B Normalized dose-response relationships of 4-MBC, α-zearalenol, di-*n*-butyl phthalate (DnBP), progesterone, and prostaglandin E1 (PGE1). Dashed lines: EC_{50} and EC_{02}.

C EC_{50} values for the EDCs (3-BC, OD-PABA, n-NP, HMS, 4-OP: $n = 3$; 4-MBC: $n = 4$; α-zearalenol, TCS, DnBP: $n = 5$; BP-3: $n = 6$). We failed to determine the EC_{50} for 4,4′-DDT. Gray arrow heads: EC_{02}; red arrow heads: maximal reported concentrations in human blood (Table 1).

D Ca^{2+} signals evoked by pico- to nanomolar 4,4′-DDT concentrations.

Data information: All values are given as mean ± SD.

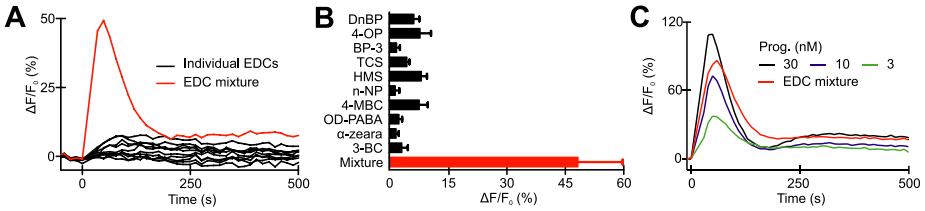

Figure 5. EDCs cooperate to evoke Ca^{2+} responses.

A Ca^{2+} signals evoked by DnBP, 4-OP, BP-3, TCS, HMS, n-NP, 4-MBC, OD-PABA, α-zearalenol, and 3-BC alone (concentration = EC_{02}, black) and by a mixture of the chemicals, containing each chemical at its EC_{02} concentration (red).

B Mean Ca^{2+} signal amplitudes evoked by individual EDCs and by the mixture used in (A) ($n = 3$). All values are given as mean ± SD.

C Ca^{2+} signals evoked by different progesterone concentrations in comparison with the Ca^{2+} response evoked by the EDC mixture used in (A).

EDCs have not been evaluated for their action in humans [16,19]. This deficit has been largely due to the lack of suitable models or procedures to systematically test large numbers of chemicals. Here, we introduce a medium-throughput assay that allows the rapid test of hundreds to thousands of chemicals for their potential to interfere with human sperm function. We trust that this new tool will greatly facilitate evaluating these chemicals with respect to their threat for human reproduction.

Materials and Methods

Detailed Material and Methods are provided in the Supplementary Materials and Methods. Human and mouse sperm were prepared as described [2,38]; changes in $[Ca^{2+}]_i$ and pH_i were measured in human sperm loaded with the Ca^{2+} indicator Fluo-4 and the pH_i indicator BCECF, respectively, in 384-microtiter plates in a fluorescence plate reader (Fluostar Omega, BMG Labtech, Germany) at 30°C [2,13]. Mouse sperm were loaded with the Ca^{2+} indicator Cal-520 (5 μM) (ATT Bioquest, USA) and changes in $[Ca^{2+}]_i$ were measured in a rapid-mixing device (SFM-400; Biologic, France) in the stopped-flow mode [38].

Whole-cell recordings were performed as described [2,13]. Seals between pipette and sperm were formed at the cytoplasmic droplet or the neck region. Monovalent currents were recorded in HS solution, containing Ca^{2+} and Mg^{2+}, and in Na^+-based divalent-free (NaDVF) bath solution. The pipette (10–15 MΩ) solution contained (in mM): 130 Cs-aspartate, 50 HEPES, 5 EGTA, and 5 CsCl adjusted

to pH 7.3 with CsOH. The osmolarity of intra- and extracellular solutions was ~320 mOsm.

For motility experiments, the flagellar beat of head-tethered sperm was recorded under an inverted microscope at 37°C. Flagellar beat asymmetry and frequency were analyzed by MATLAB (Mathworks, Germany). Acrosomal exocytosis was assessed by PNA-FITC staining.

Data are given as mean ± standard deviation (SD); n = number of experiments.

Supplementary information for this article is available online: http://embor.embopress.org

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Author contributions
NES and TS conceived the project. CS, AM, DLE, LA, CB, KA, AR, HF, BW, MB, DW, and TS designed and performed experiments. AM and TS wrote the manuscript. All authors revised and edited the manuscript.

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Conflict of interest
The authors declare that they have no conflict of interest.

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